Evaluation of Urinary Plasminogen Activator, Its Receptor, Matrix Metalloproteinase-9, and von Willebrand Factor in Pancreatic Cancer

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ABSTRACT

Purpose: Pancreatic cancer remains a devastating problem with the majority of patients succumbing to death from this disease. A hallmark of pancreatic cancer is the loss of basement membrane that may be attributed to the action of urinary plasminogen activator (uPA) and matrix metalloproteinase-9 (MMP-9). These enzymes are also implicated in angiogenesis. uPA and microvessel density have been shown to be good prognostic indicators for breast and colon cancer. MMP-9 and microvessel density have not been investigated in pancreatic cancer. We have therefore investigated by immunohistochemistry: (a) frequency of uPA expression and its receptor uPAR and the site of synthesis of uPA by in situ hybridization (ISH); (b) MMP-9 and its coexpression with uPA; (c) microvessel density as determined by von Willebrand factor staining and its relationship to uPA and MMP-9 expression; and (d) correlation of these parameters with survival.

Experimental Design: Archival paraffin sections of 27 pancreatic tumors were semiquantitatively investigated by immunohistochemistry using the following antibodies: (a) monoclonal antibodies (MAbs) uPA1 and uPA2 (3689 and 394, respectively); (b) MAb uPAR, (no. 3932); (c) MAAb MMP-9 (no. 936); and (d) rabbit anti-F8RA/vWF. ISH was performed using a uPA cDNA.

RESULTS: Both uPA antibodies revealed overexpression of uPA (93%) often with uniform staining of tumor cells. uPAR and MMP-9 showed focal staining in only 52 and 37% of tumors, respectively. Morphologically normal appearing ductal cells in close proximity to tumors overexpressed uPA in contrast to distally located normal cells (P < 0.001). uPA staining was also investigated in pancreatic intraepithelial neoplasia (PanIN) lesions. PanIN1A/B staining for uPA was seen in 8 cases (30%), that for PanIN2 in 19 cases (70%), and for PanIN3 in 12 cases (44%). Lumen of microvessels in the tumor stroma also revealed staining of uPA in 10 cases (37%). ISH experiments revealed the presence of uPA mRNA not only in the cytoplasm of tumor cells but also in adjacent normal appearing ducts as well as in PanIN lesions. Patients with overexpression of uPA, uPAR, or MMP-9 had a trend toward poorer survival than those who did not express it. Microvessel density did not show any significant relationship with uPA, uPAR, and MMP-9 expression and survival.

CONCLUSIONS: We conclude that uPA and MMP-9 are potential prognostic indicators in pancreatic cancer, whereas microvessel density may not be one. This study confirms our previous observation that uPA is made by the tumor cells themselves. Presence of uPA in vessels of tumor stroma suggests that uPA is in circulation, and its measurement and that of MMP-9 in the blood of these patients may aid in prognosis. Patients showing overexpression of uPA and MMP-9 have a trend toward shorter survival time.

INTRODUCTION

Pancreatic cancer continues to be a formidable disease and is associated with a very high incidence of fatality. Its early signs are indistinct and prone to confusion, and it is therefore considered to be a silent malignancy. Patients most commonly present with advanced disease, many of them with local or distal dissemination, and their overall 5-year survival rate is <5% (1, 2). Extensive research has focused on finding reliable molecular markers that would be both specific and sensitive. However, many of the markers studied were not clinically useful because of poor specificity and/or sensitivity (2).

An equally important effort is being directed toward identifying agents that may be directly involved in the molecular mechanisms responsible for both local invasion and distant metastasis, with the hope that some of these may also have diagnostic and/or prognostic significance. A hallmark of pancreatic cancer is the loss of continuity of type IV collagen in the basement membrane and the absence of basement membrane proteoglycans (3). In view of this, numerous investigations have dealt with proteases capable of breaking down components of
the basement membrane and ECM\(^2\), thereby facilitating tumor cell dissemination via capillaries and/or lymph channels. Of particular significance in this respect are the plasminogen activator system (uPA, uPAR, inhibitors PAI-1 and PAI-2), and the MMPs. The enzyme uPA converts the inactive proenzyme plasminogen into the potent general protease plasmin. Plasmin is now known to activate the precursors of MMPs, thereby greatly extending the range of susceptible substrates (4). Expression of uPA, its receptor, and inhibitor PAI-1 has been correlated with progression and survival in many cancers (5–7). The complex interaction between these components, however, is not yet fully understood (8, 9). In recent years, it has become clear, however, that the initial view of the role of proteases, as agents mainly responsible for clearing the path for invading cancer cells, was too simplistic. There is now good evidence to show that metalloproteinases, once activated, are capable of initiating far reaching reactions by activating, among others, transforming growth factor \(\beta\), vascular endothelial growth factor, tumor necrosis factor \(\alpha\), and liberating angiostatin from plasminogen (10, 11).

uPA has been shown to mediate the release of ECM bound fibroblast growth factor 2, a known mediator of angiogenesis (12), through the action of plasmin, whereas MMP-9 was shown to initiate angiogenesis by mobilizing the vascular endothelial growth factor from the ECM in a mouse pancreatic islet \(\beta\)-cell carcinogenesis model (13). Similarly, uPA from the conditioned medium of a pancreatic cancer cell line was shown to generate angiostatin, presumably indirectly (14), whereas MMP-9 can directly cleave plasminogen into angiostatin in vitro (11).

Two studies in the past have addressed the role of the uPA system in pancreatic cancer. Takeuchi et al. (15) found by immunohistochemical means that increased expression of uPA seemed to correlate with shorter survival and also made the important observation that the strong expression of PAI-2 significantly correlated with longer survival. Cantero et al. (16) observed that concomitant expression of uPA and uPAR correlated with shorter survival times.

Because both uPA and MMPs, particularly MMP-9 are known to be involved in the breakdown of ECM, resulting in tumor cell dissemination and may also regulate angiogenesis (17), we undertook to investigate by immunohistochemistry and by in situ hybridization: (a) the frequency of expression of uPA and its receptor, as well as the site of its biosynthesis; (b) the frequency of expression of the metalloproteinase, MMP-9, and its possible coexpression with uPA; and (c) the relation of the expression of these to the degree of vascularization, expressed as microvessel density, by visualizing the vascular endothelial marker, vWF. We report here that uPA is expressed almost universally in pancreatic cancer, and its origin is both in the malignant cells lining the ducts as well as those that arise from the acini. The receptor for uPA was expressed infrequently suggesting that, at least in this disease, the pathophysiological function of uPA may not be contingent upon interaction with its receptor. An unexpected finding was that morphologically normal ducts, when in the vicinity of cancerous foci as well as blood vessels in the tumor stroma, frequently express uPA. MMP-9 expression was also less frequent, but patients who did overexpress MMP-9 have a tendency for poorer survival than those who do not. The tumoral microvessel density showed no obvious correlation with uPA or MMP-9 or with survival.

**MATERIALS AND METHODS**

**Tissue Samples**

Twenty-seven archival pancreatic tissue specimens were analyzed. The tumor specimens consisted of 21 sections from the head, 5 from the body and tail, and 1 from the tail of the pancreas. The tumors of the head of the pancreas comprised 9 poorly differentiated adenocarcinomas, 6 that were moderately well differentiated of ductal type, and 6 poorly to moderately differentiated ductal type adenocarcinomas. The 5 tumors of the body and tail were moderately differentiated adenocarcinomas. The single tumor of the tail was a moderately to well-differentiated adenocarcinoma. Each pancreatic tumor specimen was reviewed by pathologists (R. M. P., D. T.) and stratified according to the PanIN classification (2, 18).

**Antibodies**

MAbs directed toward different catalytic domains of the \(\beta\) chain of uPA \# 3689 (uPA\(_1\)) and 394 (uPA\(_2\)), no. 3932 to the uPA receptor domain II, were obtained from American Diagnostica (Greenwich, CT). The specificities of these antibodies have been previously described by us (19). The uPA\(_1\) antibody is strictly active site specific, whereas the uPA\(_2\) antibody covers a larger domain that includes the active site. The MAb to MMP-9 (936) was purchased from R&D Systems, Inc. (Minneapolis, MN). According to the manufacturer, this antibody binds both pro and active forms of human MMP-9 and shows no cross reactivity with recombinant human MMP-1, MMP-2, or MMP-3. Rabbit anti-F8RA-vWF (factor VIII-related antigen-vWF) antibody was purchased from Dako (Carpenteria, CA).

**Other Reagents**

The secondary universal biotinylated antibody and the 3,3'-diaminobenzidine substrate kit for use in the Ventana-automated stainer were obtained from Ventana Medical Systems (Tucson, AZ). Superblock blocking buffer in TBS was purchased from Pierce Chemical Co. (Rockford, IL). Trypsin was purchased from Life Technologies, Inc. (Grand Island, NY). Triton X-100 and Tween 20 were purchased from Sigma (St. Louis, MO). A pretreatment kit for paraffin-embedded sections and the hybridization buffer used for ISH studies was from Vysis, Inc. (Downers Grove, IL). Nuclease-free water was from Ambion (Austin, TX). RNAase block solution, the biotinylated antiDIG MAb, streptavidin-alkaline phosphatase, and Fast Red substrate were from Innogenex (San Ramos, CA). DIG-UTP labeling kit for DNA, pepsin, proteinase K, EcoRI, RNAase, and Cot1 DNA were purchased from Roche Molecular Biochemicals (Indianapolis, IN).

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\(^2\) The abbreviations used are: ECM, extracellular matrix; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; vWF, von Willebrand Factor; PanIN, pancreatic intraepithelial neoplasia; MAb, monoclonal antibody; TBS, Tris-buffered saline; ISH, in situ hybridization; DIG, digoxigenin; DIG-UTP, DIG-11-dUTP.
Immunohistochemistry

The details of the procedure have been described previously (19). Briefly, the deparaffinized sections were trypsinized (0.05% trypsin with 0.05% Triton X-100 in TBS) for 20 min, blocked with 10% goat serum in Superblock, and each section incubated separately with MAbS (a) uPA$_{A}$, 20 µg/ml, (b) uPA$_{B}$, 20 µg/ml, (c) uPAR, 10 µg/ml, (d) MMP-9, 8 µg/ml, and (e) a 1:50 dilution of Dako rabbit anti-vWF, (all diluted in Superblock) at 4°C for 18–24 h. After washing the slides four to five times (15 min each) with Trition-TBS, they were processed in the Ventana-automated stainer as described previously (19). The immunoperoxidase-3,3′-diaminobenzidine-stained slides were subsequently counterstained with hematoxylin and mounted with a coverslip. Normal kidney sections were used as positive controls for uPA. Normal colon tissue known not to express uPA, and uPA$_{A}$ antibodies absorbed with immobilized uPA were used as negative controls as described previously (19).

ISH

Labeling of uPA Probe. To determine whether the uPA present in cancer cells is synthesized by these cells themselves or is derived from stromal elements, ISH experiments using uPA-cDNA were performed on 6 tumors and 2 normal sections. Preparation and purification of the uPA cDNA have been previously described by us (19). Two to three µg of EcoRI linearized uPA cDNA were labeled with DIG-UTP using random primers according to the manufacturer’s directions (Roche Molecular Biochemicals). The labeled probe was purified using Microcon EZ and Microcon PCR (Millipore Corporation, Bedford, MA) spin columns. Efficiency of labeling was checked by a dot blot assay and alkaline phosphate conjugate of rabbit anti-DIG.

Labeling of Control Sense uPA Probe. The sense strand of uPA was labeled with DIG-UTP using two uPA sense primers rather than random hexamers that were used for labeling of the uPA cDNA as a positive control. Both primers were synthesized by the Biopolymer Facility at this institute. Primer 1 corresponding to nucleotides 282–303 of the uPA cDNA had the following sequence 5′-CCTGCTATGAGGGGAATGGT-CAC-3′. Primer 2 had a 30-mer sequence of uPA corresponding to nucleotides 450–470 to which a 17-mer sequence of PUC19 (nucleotides 418–434) in the poly linker cloning site was added on as follows: 5′-GCAGGAACCCAGACACCGGAGGCGACCTGTAGTTGATGAGTCCAC-3′. The underlined sequence representing the sequence in the vector PUC19. Two µg of the plasmid with the uPA insert were linearized with EcoRI, denatured, labeled with DIG-UTP in presence of 10 µmol of the above primers and Klenow enzyme according to the procedure described by the manufacturer of the DIG-UTP labeling kit (Roche Molecular Biochemicals). The labeled probe was purified as described above.

Preparation of Tissue Sections for Hybridization. The paraffin sections were baked overnight at 60°C followed by incubation of the slides at −20°C for 4 h. They were then deparaffinized in xylene and dehydrated in 100% ethanol, and subsequently digested with 10 µg/ml proteinase K in 50 mM TBS (pH 7.9) containing 5 mM EDTA at 37°C for 10 min. After the protease treatment, the slides were washed in TBS and fixed in 10% formalin in 1× SSC buffer [0.15 M NaCl-0.015 M sodium citrate (pH 7.0)] for 10 min and then washed twice with SSC buffer.

The endogenous RNAase activity was blocked with a 0.2% solution of RNAase block solution (Innogenex) at 37°C for 10 min. The slides were then rinsed in 2× SSC buffer twice and dehydrated in graded cold alcohol of 70, 90, and 100% (−20°C) and subsequently denatured in 80% formamide in 2× SSC at 80°C for 5 min. Subsequently, they were dehydrated in cold (−20°C) graded ethanol and used for hybridization.

Preparation of Probes for Hybridization. Two µg of DIG-labeled uPA cDNA or control sense uPA DNA in 50 µl of SSC buffer were mixed with 30 µl of Cot1 DNA (1 mg/ml) and 8 µl of 3 m cold sodium acetate followed by the addition of 5 volumes (400 µl) of cold (−20°C) absolute ethanol, gently mixed, and left to precipitate at −20°C for 1 h. The precipitated DNA was centrifuged, and the pellet washed twice with 70% cold ethanol and air-dried. The DNA was then dissolved in 80 µl of hybridization buffer (Vysis, Inc.), denatured at 75°C for 5 min, and cooled to room temperature and immediately used for hybridization.

Hybridization and Detection. Fifteen µl of the above probe (375 ng of DNA) in the hybridization buffer were applied to the formamide-denatured slides and covered with glass coverslips. Care was taken to exclude all air bubbles. The slides were then incubated in a moist chamber at 37°C for 18–20 h. After incubation, the coverslips were removed in coplin jars containing 2× SSC with 0.05% Tween 20. The slides were consecutively washed with 2×, 1×, and 1/2× SSC buffer and treated with a blocking solution (Roche Molecular Biochemicals), incubated with biotinylated anti-DIG MAb (Innogenex) followed by streptavidin alkaline phosphate incubation, and color development with Fast Red according to the manufacturer’s instructions (Roche Molecular Biochemicals). A control slide treated with RNAase [100 µg/ml in 2× SSC (pH 7.0)] and incubated at 37°C for 30 min as well as a slide hybridized with sense uPA probe was included in every set of experiments. Subsequent to color development, the slides were mounted with a coverslip using an aqueous mount.

Microvessel Density Determination

Assisted by a pathologist, intratumoral microvessel density was determined manually by counting vessels stained with F8RA-vWF antibody as described in detail by Weidner and Folkman (20), except in this case an average of counts in 15 fields (×200 magnification with 0.74 mm²/field) was determined. We used the same criteria for identifying microvessels as that of Weidner and Folkman (20).

RESULTS

Taken together, 27 pancreatic tumors were examined for uPA, uPAR, MMP-9, and vWF staining. Table 1 shows the relative staining patterns using antibodies directed against the above antigens.

uPA Immunolocalizes to the Tumor Cells. As expected, uPA expression is nearly ubiquitous, as both uPA$_{A}$ and uPA$_{B}$ antibodies detect the enzyme antigen in 93% of the tumors examined, with only 2 tumors failing to express it (Table 1). There were occasional differences in the degree of expression of
**Table 1** Immunohistochemical analyses of pancreatic tumors for staining with Mabs to uPA, uPAR, MMP-9, and rabbit anti-vWF: correlation of uPA staining with PanIN and adjacent normal lesions

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Tumor size (cm)</th>
<th>Location</th>
<th>PanIN classification/differentiation</th>
<th>uPA 1</th>
<th>uPA 2</th>
<th>N°</th>
<th>1A/B</th>
<th>2</th>
<th>3</th>
<th>4*</th>
<th>uPAR</th>
<th>MMP-9</th>
<th>vWF</th>
<th>Survival (mos)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Head</td>
<td>PanIN2&amp;3 invasive PD</td>
<td>3+</td>
<td>4+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Focal 3+</td>
<td>–</td>
<td>26</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>Head</td>
<td>PanIN3 invasive PD</td>
<td>4+</td>
<td>4+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Focal 3+</td>
<td>–</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>2.5</td>
<td>Head</td>
<td>Invasive adenocarcinoma PD</td>
<td>2+</td>
<td>4+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Focal 3+</td>
<td>–</td>
<td>19</td>
<td>&gt;36 alive</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Body and Tail</td>
<td>Invasive perinugal adenocarcinoma WD</td>
<td>3+</td>
<td>4+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Focal 3+</td>
<td>–</td>
<td>16</td>
<td>13</td>
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<tr>
<td>5</td>
<td>2.3</td>
<td>Body and Tail</td>
<td>in situ panIN3 lobular invasive adenocarcinoma MD</td>
<td>4+</td>
<td>3-4+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Focal 2-3+</td>
<td>–</td>
<td>11</td>
<td>32</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>Head</td>
<td>transition from PanIN2 to invasive adenocarcinoma WD to MD</td>
<td>4+</td>
<td>3-4+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Focal 3+</td>
<td>4+</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
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<td>Head</td>
<td>in situ w/PanIN3 invasive adenocarcinoma WD to MD</td>
<td>3-4+</td>
<td>2+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Focal 3+</td>
<td>–</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>8</td>
<td>2.5</td>
<td>Head</td>
<td>adenocarcinoma invasive desmoplasia</td>
<td>3-4+</td>
<td>2+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Focal 3+</td>
<td>–</td>
<td>9</td>
<td>&gt;72 alive</td>
</tr>
<tr>
<td>9</td>
<td>2.5</td>
<td>Body and Tail</td>
<td>invasive adenocarcinoma desmoplasia</td>
<td>3-4+</td>
<td>2+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>24</td>
<td>25</td>
</tr>
<tr>
<td>10</td>
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<td>Body and Tail</td>
<td>High nuclear grade with clear nuclear cell invasive adenocarcinoma PD</td>
<td>3+</td>
<td>2+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Focal 2-3+</td>
<td>31</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>Tail</td>
<td>in situ w/PanIN3 to adenocarcinoma; panIN1&amp;2 components MD</td>
<td>2+</td>
<td>3+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Focal 4+</td>
<td>–</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>5.6</td>
<td>Body and Tail</td>
<td>invasive adenocarcinoma desmoplasia</td>
<td>2+</td>
<td>3+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>11</td>
<td>0.3</td>
</tr>
<tr>
<td>13</td>
<td>3.5</td>
<td>Head</td>
<td>PanIN1 progressive to PanIN2 and invasive adenocarcinoma</td>
<td>1-2+</td>
<td>3+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>18</td>
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<tr>
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<td>1.4</td>
<td>Head</td>
<td>PanIN3 w/invasive adenocarcinoma PD</td>
<td>2+</td>
<td>2+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>16</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>*NA</td>
<td>Head</td>
<td>PanIN1 and PanIN2 WD</td>
<td>1-2+</td>
<td>2+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Focal 3+</td>
<td>–</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>16</td>
<td>4.5</td>
<td>Head</td>
<td>PanIN1 to PanIN2 to invasive adenocarcinoma; in situ component</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Focal 3+</td>
<td>–</td>
<td>37</td>
<td>&gt;60 alive</td>
</tr>
<tr>
<td>17</td>
<td>15</td>
<td>Head</td>
<td>PanIN1, mucinous papillary carcinoma w/in situ WD</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>14</td>
<td>&gt;60 alive</td>
</tr>
<tr>
<td>18</td>
<td>2.5</td>
<td>Head</td>
<td>Invasive adenocarcinoma PD</td>
<td>3+</td>
<td>3+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>37</td>
<td>&gt;6 alive</td>
</tr>
<tr>
<td>19</td>
<td>1.8</td>
<td>Head</td>
<td>PanIN2 to invasive adenocarcinoma PD</td>
<td>2+</td>
<td>3+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>Focal 3+</td>
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<td>17</td>
<td>&gt;9 alive</td>
</tr>
<tr>
<td>20</td>
<td>3.2</td>
<td>Head</td>
<td>Clear cells, PanIN2 to invasive adenocarcinoma</td>
<td>4+</td>
<td>Focal 2-3+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>24</td>
<td>&gt;25 alive</td>
</tr>
<tr>
<td>21</td>
<td>1.3</td>
<td>Head</td>
<td>Invasive adenocarcinoma, in situ component MD</td>
<td>2+</td>
<td>3+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>26</td>
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</tr>
<tr>
<td>22</td>
<td>2.5</td>
<td>Head</td>
<td>Invasive adenocarcinoma MD</td>
<td>Focal 2-3+</td>
<td>Focal 2+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>Focal 3+</td>
<td>2+</td>
<td>23</td>
<td>&gt;4 alive</td>
</tr>
<tr>
<td>23</td>
<td>5</td>
<td>Head, uncinate, body</td>
<td>PanIN3 to invasive adenocarcinoma; in situ component MD to PD</td>
<td>2+</td>
<td>2-3+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>22</td>
<td>&gt;24 alive</td>
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<tr>
<td>24</td>
<td>4.5</td>
<td>Head</td>
<td>PanIN1 to invasive adenocarcinoma, perinugal invasion MD to PD</td>
<td>1-2+</td>
<td>4+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>23</td>
<td>&gt;4 alive</td>
</tr>
<tr>
<td>25</td>
<td>1.6</td>
<td>In situ, head</td>
<td>PanIN3 to in situ, has PanIN1&amp;2 WD</td>
<td>3+</td>
<td>2-3+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Focal 1-2+</td>
<td>25</td>
<td>25</td>
<td>&gt;24 alive</td>
</tr>
<tr>
<td>26</td>
<td>3.5</td>
<td>Head</td>
<td>PanIN3; in situ and adjacent invasive MD</td>
<td>Focal 2-3+</td>
<td>2-3+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Focal 2+</td>
<td>2+</td>
<td>42</td>
<td>&gt;7 alive</td>
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<tr>
<td>27</td>
<td>5.5</td>
<td>Head</td>
<td>Invasive adenocarcinoma PD</td>
<td>Focal 3+</td>
<td>3+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Focal 2+</td>
<td>–</td>
<td>29</td>
<td>0.13 alive</td>
</tr>
</tbody>
</table>

* a N, normal ducts in the immediate vicinity of the tumor. Staining of these normal ducts (n = 14) were significantly different from those that were at a distant site (n = 16) showing no staining for uPA (P < 0.001).

* b 4 represents invasive adenocarcinoma with desmoplasia.

* c PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated; NA, not available; 1A/B, 2 and 3 represent progressive stages of pancreatic intraepithelial neoplasias.

* d +++, indicates staining of all tumor cells. vWF shows microvessel density as an average of counts in 15 fields according to the criterion used by Weidner and Folkman (19).
uPA among the tumors examined depending on the antibody used (Table 1). Moderate to intense staining was almost always observed with uPA2 antibody in the cytoplasm and the membrane of epithelial cells of the ductal lining, as well as the acini. Fig. 1, A and B, at a higher magnification, show uniform granular staining throughout the entire cytoplasm and the membrane of the cancer cells, with the basal regions of the ducts showing the strongest staining. A hitherto unreported finding was that in a significant number of cases the morphologically normal glands (14 of 16) adjacent to the frankly transformed...
ducts showed a positive reaction for uPA (P < 0.001; Fig. 2D, Table 1), whereas none of the normal glands (0 of 16) distant to the tumor were positive (Fig. 1E). A striking observation was that in 10 of 27 cases, strong staining for uPA was observed in the lumen of large blood vessels located in the stroma, proximal to the tumor (Fig. 1C). The staining is because of uPA itself trapped within the RBC mass because controls with irrelevant mouse IgG showed no staining in these vessels (Fig. 1D). Many of the tumors showed staining for uPA in stromal elements. As shown in Fig. 1A, the desmoplastic reaction revealed moderate staining of histiocytes, monocytes, and occasionally fibroblasts. Regions of perineural invasion also exhibited staining for uPA (data not shown).

**uPA Staining in PanIN Lesions.** On the basis of the premalignant characteristics, we have evaluated the expression of uPA in PanIN lesions. Specifically, we found that this marker was present in 8 cases with PanIN 1 lesions (8 of 27) as shown in Table 1. The criteria for classification of PanIN lesions we followed were according to that of Kern et al. (2) and Bardesey and Depinho (18). uPA staining was also observed in PanIN 2 (19 of 27) and PanIN 3 (12 of 27) lesions. Invasive adenocarcinomas with desmoplastic reaction almost always stained intensely for uPA.

**uPAR Is Infrequently Expressed in Tumor Cells.** Positive reaction for uPAR was found in 14 of the 27 tumors examined. The intensity of staining was weak to moderate and always focal (Table 1 and Fig. 1F). These tumors also revealed the presence of uPAR in normal cells in areas of desmoplastic reaction (data not shown). The possible significance of this finding is discussed below.

**Fig. 2** A. ISH with DIG-labeled uPA cDNA for the detection of uPA mRNA in a well to moderately differentiated ductal adenocarcinoma (case no. 6, Table 1). The Fast Red stain indicates the presence of uPA mRNA. Some histiocytes as well as fibroblasts are also stained in the stroma. Magnification: ×100. B. DIG-labeled uPA sense probe hybridization of the same tumor shown in A. Lack of Fast Red staining indicates no hybridization with uPA mRNA. Magnification: ×100. C. Presence of uPA mRNA in the stratified tumor confined to the ductules in the same tumor shown in A. An adjacent normal duct also reveals the presence of uPA mRNA (arrow). Hybridization was performed using the same probe as in A. D. ISH with DIG-labeled uPA cDNA reveals the presence of uPA mRNA in a histologically normal appearing mucinous metaplasia adjacent to the tumor (case no. 10, Table 1) indicating early gene expression of uPA in such lesions. Magnification: ×200. E. uPA mRNA is also expressed in PanIN 1 lesion as shown by hybridization of DIG-labeled uPA cDNA with the same tumor section shown in A. Arrow points to PanIN 1 lesion. Arrowheads show normal acini; L = lymphocytes. Magnification: ×100. F. ISH with DIG-labeled sense uPA probe used as a negative control for the section shown in E shows no Fast Red staining of uPA mRNA. Magnification: ×100.
uPA mRNA Predominantly Localized to Tumor Cells but not Stromal Elements. uPA ISH demonstrates that uPA is synthesized by tumor cells themselves, whereas the surrounding stromal elements do not significantly contribute any uPA mRNA. Fig. 2A shows strong expression of uPA mRNA in the cytoplasm of a well to moderately differentiated invasive adenocarcinoma of the head of the pancreas. The sense probe on the other hand shows no hybridization (Fig. 2B). In another area of the same tumor (Fig. 2C), the presence of uPA mRNA is seen in the stratified tumor confined to the ductules. A section of this tumor treated with RNAase showed no hybridization (data not shown). uPA mRNA was also strongly expressed in a mucinous metaplasia in proximity of a malignant lesion in an invasive ductal adenocarcinoma (Fig. 2D). None of the stromal elements surrounding the tumor revealed uPA-mRNA staining in the sections examined. Fig. 2E shows that uPA mRNA is also expressed in PanIN-1 lesion, whereas the control sense probe shows no hybridization (Fig. 2F), thus supporting the immunohistochemical observations. Interestingly, the two specimens that were negative for immunohistochemical staining of uPA (case nos. 16 and 17, Table 1) showed a very weak hybrid hybridization with DIG-uPA (data not shown).

MMP-9 Expression. Staining for this protease was also focal (10 of 26; Table 1) and in the case of uPAR (14 of 27; Table 1), it was weak to moderate (Fig. 1G and Table 1). Intense staining of stromal elements was observed in tumors where uPA was not expressed. MMP-9 showed up in only some of the ducts, suggesting little, if any, connection between the expression of these two entities. In a few cases, normal ductal and acinar cells also showed a positive reaction (data not shown).

Microvessel Density. Neovascularization in a well to moderately differentiated tumor is shown in Fig. 1H. The degree of neovascularization did not correlate with either PanIN lesions or the differentiation. The average density for all of the tumors had a broad range from 9 to 37 vessels/0.74 mm² of the microscopic field. The microvessel density showed no correlation with uPA, uPAR, or MMP-9 expression and survival.

Correlation with Survival. Neither uPA, nor uPAR, or MMP-9 showed a significant correlation with survival although there was a trend toward shorter survival (data not shown).

DISCUSSION

Overexpression of uPA has been a consistent finding in adenocarcinomas of the colon, lung, breast, prostate, ovaries, and melanoma (reviewed in Refs. 9, 21–27), whereas corresponding normal tissues did not express the activator. In contrast to breast or colon cancer, which have been dealt with in a large number of studies, relatively little attention has been paid to the occurrence of uPA in pancreatic cancer, except for the studies of Takeuchi et al. (15) and Cantero et al. (16) who correlated the overexpression of uPA and PAI-2 or uPAR, respectively, with shorter survival.

The data presented here document that the overexpression of uPA is an almost ubiquitous feature (93% of cases) of pancreatic cancer, as it is of all other malignancies thus far examined. In this disease, however, the pattern of cellular expression differs from other malignancies (e.g., colon cancer). In colon cancer, uPA is typically expressed uniformly, i.e., all glandular colon cancer cells show a positive antibody reaction (19), whereas in pancreatic cancer, there is a heterogeneous expression of uPA within the ducts and acini. We have observed a similar heterogeneous expression of uPA in breast cancer. A significant and, thus far, unreported observation is the presence of uPA in morphologically normal glands adjacent to the tumor. This expression of uPA suggests an early event in malignant transformation. That the antigen is not simply taken up by these morphologically normal structures from the cancerous glands is shown by the presence of uPA-mRNA in these normal looking cells. The malignant epithelium of the ductal lining and of the acini are frequent sites of uPA expression. Interestingly, the two cases that were negative for uPA antigen expression (Table 1, nos. 16 and 17) but showed a weak expression of uPA mRNA were also the ones with longest survival. A striking observation was that in many tumors, the lumen of blood vessels in the peritumoral stroma was found to be full of material reacting positively for uPA (Fig. 1C). Although we were unable to ascertain the origin of the antigen, the observation suggests that uPA from the tumors may be shed into circulation. Thus, elevated serum levels of uPA may be indicative of the presence of pancreatic cancer. Our results of overexpression of uPA in 93% of tumors is in close agreement with those of Takeuchi et al. (15), who demonstrated that uPA overexpression in 78% of patients predominates and excesses tissue plasminogen activator expression. They additionally demonstrated that patients with strong uPA expression were associated with shorter survival times.

Although we did not find any correlation with the coexpression of uPA and uPAR, Cantero et al. (16), who analyzed pancreatic cancer patients for uPA and uPAR by immunohistochemistry and Northern blot technique, found a strong coexpression of uPA with uPAR in most of the cases investigated. This concomitant overexpression of uPA and uPAR was additionally found to be associated with shorter survival. In our study, the uPA receptor was detected in only 14 of 27 patients (Table 1) and always focally, an unexpected result. Furthermore, coexpression of the receptor was not always found in the same tumor cells where uPA was expressed. It is possible that the difference in the specificity of the uPAR antibodies may have contributed to this discrepancy. According to current views, uPA is fully effective only when anchored to its receptor on the cell surface (8, 28). The present data, however, indicate that whatever the function of the enzyme may be in pancreatic cancer, binding to the receptor does not appear to be a requirement. Nonetheless, in our studies, both uPA and uPAR showed a trend toward shorter survival times (Table 1).

Two important observations not reported previously have been made in this study: (a) there is a significant overexpression of uPA in histologically normal ducts/acini that are in the immediate vicinity of the tumor; and (b) blood vessels in the tumor stroma reveal uPA. These observations strongly suggest that a search for the presence of the transformed (uPA-positive)
phenotype in the absence of demonstrable cancer could be used as a criterion for early intervention.

The ISH in this study shows again that the site of uPA expression in the cancer cells is also the site of its synthesis. It is important to point out this fact because the view has been repeatedly advocated that uPA is produced by interstitial stromal elements and is then taken up in a paracrine fashion by receptors located on neighboring cancer cells (29). In an earlier study, we have conclusively shown that in colon cancer, it is the cancer cells themselves that are the site of uPA production (19). There is no doubt, however, that interstitial elements also produce uPA, as was seen also in this study. As seen in the ISH for uPA, the adjacent normal ducts reveal the presence of uPA mRNA (Fig. 2D), indicating that these cells are transformed but not yet malignant. This finding suggests that uPA expression is an early event in malignant tumor formation.

The multifunctional role of metalloproteinases in cancer is the subject of much current research. We chose to investigate MMP-9 on account of its reported ability to induce angiogenesis in pancreatic islet β-cell tumorigenesis, as well as to generate angiotatin by proteolytic cleavage of plasminogen. We detected MMP-9 in only 10 of the 27 cases investigated and found no correlation with its occurrence and the intratumoral microvessel density or uPA expression. However, the occurrence of MMP-9 showed a trend toward negative correlation with survival. Evidently, other effects of MMP-9 must outweigh its role in angiogenesis. Schmaldfelt et al. (30) have shown that pro-MMP-2 and pro-MMP-9, as well as uPA and PAI-1 antigen levels were low in benign tumors but increased significantly from low malignant potential to advanced ovarian cancers with highest levels in metastasis into the momentum. However, they did not find any correlation between uPA and MMP-9, an observation similar to ours in pancreatic cancer.

We chose to use F8RA/vWF antibody rather than a CD-31 antibody in microvessel density determination because Weidner and Folkman (20) have found this antibody highly specific for endothelial cells. However, we found no correlation between the microvessel density and uPA, MMP-9, or survival of patients. They have additionally shown that intratumoral microvessel density was found to have an independent prognostic significance when compared by multivariate analysis with traditional markers in breast cancer, as well as many other types of cancer reviewed by them (20). Our results suggest that in pancreatic cancer, tumor dissemination and metastasis may be facilitated by preexisting vessels, whereas uPA and MMP-9 do not appear to play a role in pancreatic tumor angiogenesis. At least in pancreatic islet β-cell carcinogenesis in the mouse, uPA does not have a role in angiogenesis (13).

This study confirms our earlier studies and adds four significant findings to the existing knowledge about pancreatic cancer. (a) uPA expression is nearly ubiquitous and is a hallmark of malignant transformation. (b) uPA expression is an early event in this process as shown by its appearance in morphologically normal ductal cells adjacent to frankly transformed ones. (c) Wherever the presence of uPA is evidenced by immunostaining, ISH shows that it is also its site of expression, a finding that contradicts current claims (29, 30). (d) The presence of uPA in blood vessels in the tumor stroma suggests that it is in circulation, and therefore, its measurement may be useful in early intervention.

REFERENCES

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Evaluation of Urinary Plasminogen Activator, Its Receptor, Matrix Metalloproteinase-9, and von Willebrand Factor in Pancreatic Cancer

Shashi R. Harvey, Thelma C. Hurd, Gabor Markus, et al.


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